Review

The Anticancer Drug Ellipticine Activated with Cytochrome P450 Mediates DNA Damage that Determines its Pharmacological Efficiencies: Studies with Knockout Mouse Models and Pure Enzymes

Marie Stiborová [1,*], Věra Černá [1], Michaela Moserová [1], Iveta Mrízová [1], Volker M Arlt [2] and Eva Frei [3]

[1] Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, CZ-12843, Prague 2, Czech Republic; E-Mails: vera.cerna@natur.cuni.cz (V.Č.), michaela.moserova@natur.cuni.cz (M. M.), iveta.vranova@natur.cuni.cz (I.M.)

[2] Analytical and Environmental Sciences Division, MRC-PHE Centre for Environmental & Health, King's College London, 150 Stamford Street, London SE1 9NH, United Kingdom; E-Mail: volker.arlt@kcl.ac.uk (V.M.A.)

[3] Division of Preventive Oncology, National Center for Tumor Diseases, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, E-Mail: eva.frei@t-online.de (E.F.)

* Author to whom correspondence should be addressed; E-Mail: stiborov@natur.cuni.cz; Tel.: +420 221951285; Fax: ++420 221951283.

Received: / Accepted: /Published:

Abstract: Ellipticine is a DNA-damaging agent acting as a prodrug whose pharmacological efficiencies and genotoxic side effects are dictated by activation with cytochrome P450 (CYP). Over the last decade we have gained extensive experience in using pure enzymes and various animal models that helped to identify CYPs metabolizing ellipticine. In this review we focus on comparison between the *in-vitro* and *in-vivo* studies and show a necessity of both approaches to obtain valid information on CYP enzymes contributing to ellipticine metabolism. The discrepancies were found between the CYP enzymes activating ellipticine to 13-hydroxy- and 12-hydroxyellipticine generating covalent DNA adducts and those detoxifying this drug to 9-hydroxy- and 7-hydroellipticine *in vitro* and *in vivo*. *In vivo*, formation of ellipticine-DNA adducts is dependent not only on expression levels of CYP3A, catalyzing ellipticine activation *in vitro*, but also on those of CYP1A that oxidize ellipticine *in vitro* mainly to the detoxification products. The finding showing that cytochrome b_5 modulates patterns of ellipticine metabolites formed by CYP1A1/2 and 3A explained this paradox. Cytochrome b_5 alters the ratio of ellipticine metabolites generated by CYP1A1/2 and 3A4. Whereas formation of detoxification ellipticine metabolites by CYP1A and 3A are either decreased

or not changed with cytochrome b_5, formation of activation metabolites and ellipticine-DNA adducts increased considerably. We demonstrate that (i) ellipticine pharmacological effects mediated by covalent ellipticine-derived DNA adducts are dictated by expression levels of CYP1A, 3A and cytochrome b_5, and its own potency to induce these enzymes in tumor tissues, (ii) the animal models, where levels of CYPs are regulated both by their knockout and/or induction are appropriate to identify CYPs metabolizing ellipticine *in vivo*, and (iii) extrapolation from *in-vitro* data to situation *in vivo* is not always possible, confirming the need for these animal models.

Keywords: Anticancer drug ellipticine; Cytochrome P450 mediated DNA-damage; Covalent DNA adducts; Enzymes metabolizing ellipticine *in vitro* and *in vivo*

1. Introduction

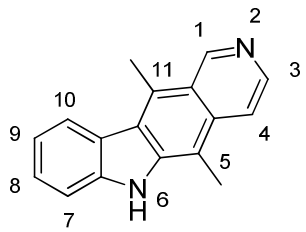
A plant alkaloid ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole, Figure 1) found in several Apocynaceae plants and its derivatives are efficient anticancer compounds that function through multiple mechanisms participating in cell cycle arrest and initiation of apoptosis (for a summary see) [1-6]. Ellipticine was found (i) to arrest cell cycle progression due to modulation of levels of cyclinB1 and Cdc2, and phosphorylation of Cdc2 in human mammary adenocarcinoma MCF-7 cells, (ii) to initiate apoptosis due to formation of toxic free radicals, stimulation of the Fas/Fas ligand system and modulation of proteins of Bcl-2 family in several tumor cell lines, and (iii) to induce the mitochondria-dependent apoptotic processes (for a summary see) [3,4].

Several studies also demonstrated that p53 tumor suppressor protein is involved in ellipticine-mediated induction of cell cycle arrest and apoptosis [7-13]. Ellipticine inhibits p53 protein phosphorylation by a selective inhibition of CDK2 kinase in Lewis lung carcinoma and human colon cancer cell line SW480 [7], and this effect on p53 correlated with cytotoxic activity of ellipticine [7]. Treatment of Saos 2 cells transfected with mutant p53 with ellipticine restored the transactivation function of p53, resulting in the induction of p53-responsive $p21^{Waf1}$ and *MDM2* genes at protein levels and activation of a p53-responsive luciferase reporter [12]. The results found in the study of Sugikawa and coworkers [12] indicate that ellipticine induces a shift of mutant p53 conformation towards wild-type and this activity is not caused by its function as an inhibitor of topoisomerase II, which is one of the DNA-damaging effects of ellipticine (for a summary see [1-6]). More importantly, ellipticine can even activate mutant p53 and induces $p21^{Waf1}$ and *MDM2* gene expression *in vivo*, in nude mouse tumor xenografts [13].

Ellipticine also modulates p53 nuclear localization in HCT116 colon cancer cells. It elevated the nuclear localization of endogenous p53 and exogenous mutant p53 with a resultant increase in the transactivation of the p21 promoter. Nuclear localization of p53 is frequently the consequence of a genotoxic stress by compounds inducing DNA damage (*i.e.* inhibitors of topoisomerase II)[14]. The ellipticine-mediated abundance of nuclear p53 was not associated with an increase in DNA double strand breaks. Therefore, this effect of ellipticine seems not to be dependent on the mechanism mediated by topoisomerase II inhibition, but on another genotoxic stress [15]. Ellipticine also activates the p53

pathway in glioblastoma cells; its impact on these cancer cells depends on the p53 status [11]. In a U87MG glioblastoma cell line expressing wild-type p53, ellipticine provoked an early G0/G1 cell cycle arrest, whereas in a U373 cell line expressing a p53 mutant it induced arrest in S and G2/M phases of the cell cycle [11].

Figure 1. Ellipticine.



All studies investigating the mechanism of ellipticine antitumor action indicate complex pathways leading to cancer cell death by this drug. Chemotherapy-induced cell cycle arrest and induction of apoptosis were shown to frequently result from DNA damage caused by exposure to a variety of chemotherapeutics including ellipticine. In addition, a genotoxic stress resulting from several DNA-damaging agents including ellipticine increases levels of nuclear p53 [14,15], the tumor suppressor protein known to be involved in ellipticine-mediated induction of cell cycle arrest and apoptosis [7-13]. These findings suggest that a role of ellipticine as a DNA-damaging agent is crucial for its cytotoxic effects.

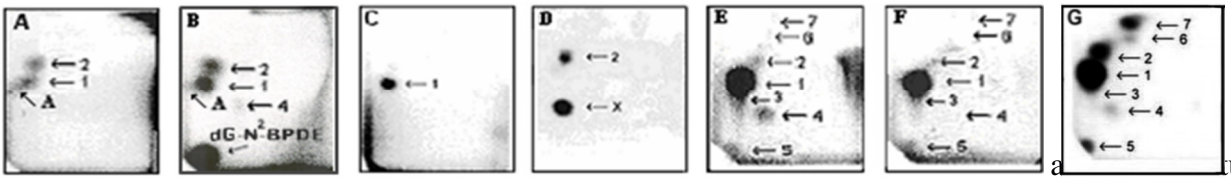
2. DNA-damaging mechanisms of ellipticine cytotoxicity to cancer cells

Of the DNA-damaging mechanisms of ellipticine's biological effects, intercalation into DNA [5,16-19] and inhibition of DNA topoisomerase II activity [5,20,21] were considered to be most important. However, we showed that this antitumor agent causes also damage to the structural integrity of DNA through covalent binding, by forming covalent DNA adducts after its enzymatic activation with cytochromes P450 (CYP) and peroxidases [1-4,22-31]. Moreover, cytotoxicity of ellipticine to several cancer cells sensitive to this drug such as HL-60 promyelocytic leukemia and T-cell leukemia CCRF-CEM cells, glioblastoma U87MG cells, neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines, thyroid cancer BHT-101, B-CPAP and 8505-C cells and breast adenocarcinoma MCF-7 cells corresponds to levels of ellipticine-derived DNA adducts generated after its enzymatic activation in most these cells [32]. This indicates that covalent modification of DNA by the reactive species generated with enzyme-mediated bioactivation of ellipticine is one of the most important mechanisms responsible for ellipticine cytotoxicity in these cancer cells. Based on these results, we suggest that ellipticine acts as a prodrug, which is metabolically activated to reactive species forming covalent DNA adducts causing genotoxic stress. Therefore, information on which enzymes are involved in the metabolism of ellipticine is critical for identification of ellipticine pharmacological effects. Several *in vitro* and *in vivo* approaches have been developed to study a role of specific CYP and peroxidase enzymes in ellipticine metabolism.

Over the past 10 years we have gained extensive experience in using the pure enzymes and the various animal models to study the ellipticine metabolism. During these studies, ellipticine was found to

be oxidized by CYP and peroxidase enzymes to both electrophilic species forming covalent DNA adducts detected by ^{32}P-postalbeling (Figure 2) and to detoxification metabolites [1-4,23-31,33-42]. Moreover, we characterized reactions leading to their formation.

Figure 2. Autoradiographs of thin layer chromatography (TLC) maps of ^{32}P-labeled digests of calf thymus DNA reacted with ellipticine activated by hepatic microsomes from wild-type (WT) mice (**A**), with those of Hepatic Cytochrome P450 Reductase Null (HRN) mice treated with benzo[a]pyrene (BaP) (**B**), from calf thymus DNA reacted with 13-hydroxyellipticine (**C**) [22] and 12-hydroxyellipticine (**D**) [23] of DNA from livers of WT (**E**) and HRN (**F**) mice treated *i.p.* with 10 mg ellipticine/kg body weight [39] and of DNA from liver DNA of Wistar rats treated *i.p.* with 40 mg ellipticine per kilogram body weight (**G**) [24,28]. Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. Adduct spots 1-7 and A correspond to the ellipticine-derived DNA adducts. Besides adduct 2 formed by 12-hydroxyellipticine, another strong adduct (spot X in panel D), which was not found in any other activation systems or *in vivo* was generated.



studies investigating the ellipticine metabolism and show a necessity of both approaches to obtain valid information on CYP enzymes participating in this process.

3. Metabolism of ellipticine by CYP, peroxidase and conjugation enzymes to activation and detoxification metabolites *in vitro*

Utilizing numerous *in vitro* systems such as subcellular microsomal fractions and cells in culture expressing CYPs, isolated CYPs reconstituted with other components of the mixed-function-oxidase system [NADPH:CYP reductase (POR), cytochrome b_5], and recombinant CYPs, the human, rat, rabbit, and mouse CYP enzymes were found to oxidize ellipticine. Up to five metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine, and ellipticine N^2-oxide (Figure 3), and at least two major ellipticine DNA-adducts were generated by these enzymatic systems [3,22,24-27,29-31,39,40].

7-Hydroxy- and 9-hydroxyellipticine are considered to be the detoxification metabolites of ellipticine, because they are efficiently excreted by experimental animals [43,44]. 13-Hydroxy- and 12-hydroxyellipticine are the active metabolites, because they are reactive compounds forming spontaneously ellipticine-13-ylium and ellipticine-12-ylium, which can generate two major deoxyguanosine adducts in DNA (see adduct spots 1 and 2 in Figure 2 and the proposed structures of these DNA adducts in Figure 3) [2-4,22,23,25-27,30,31,40]. In addition, ellipticine N^2-oxide should be considered as another active ellipticine metabolite, because it rearranges to 12-hydroxyellipticine [22], by the Polonowski rearrangement [45] (Figure 3). All these results suggest that the enzymes activating

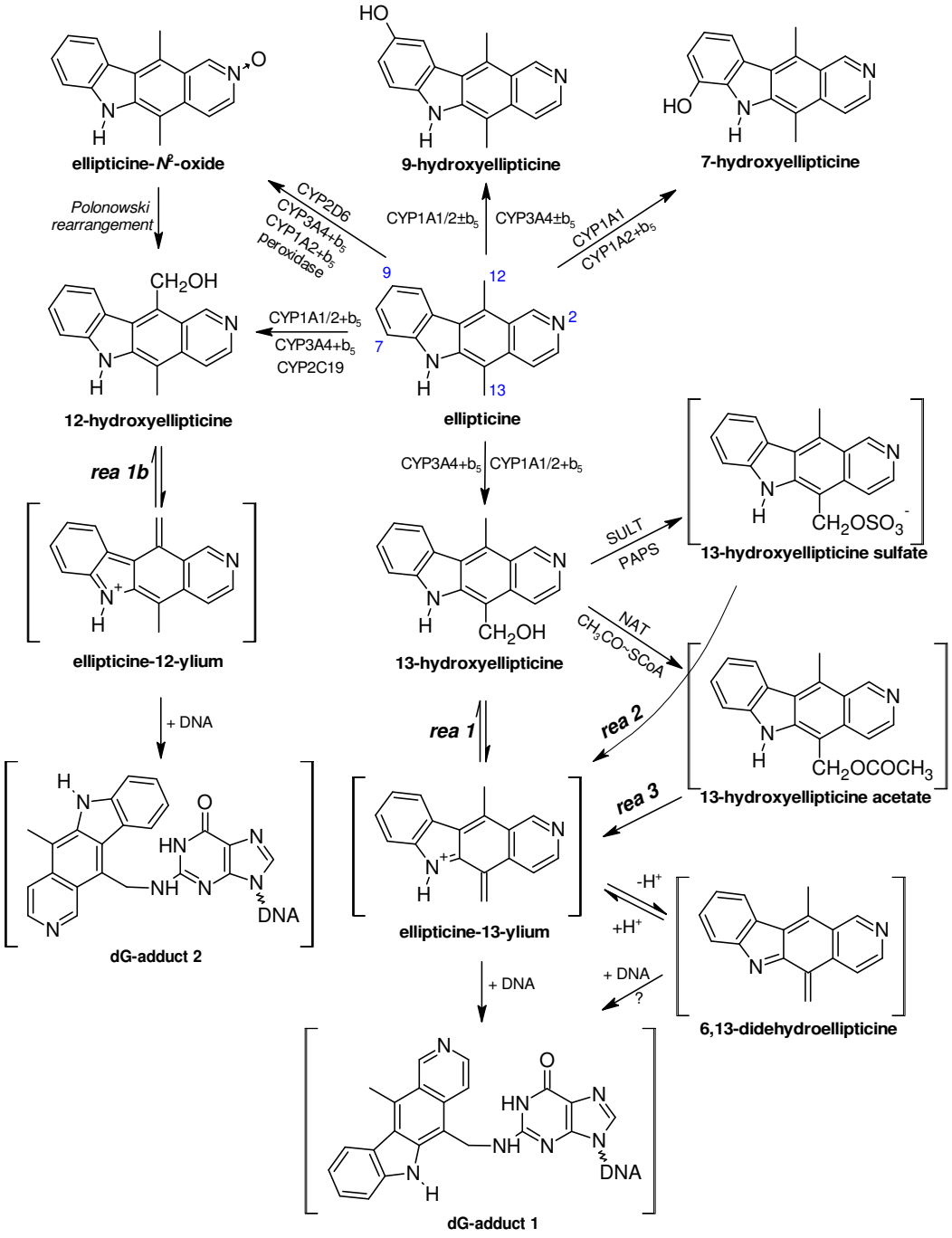
or detoxifying ellipticine are crucial for its pharmacological effects. Thus, the identification of enzymes necessary for ellipticine metabolism is of great importance.

Using a panel of human recombinant CYPs, inhibitors of these enzymes in human hepatic microsomes and correlation analyses, the role of individual CYPs in the formation of ellipticine metabolites was identified [22,25,26,31]. Human recombinant CYP1A1 and 1A2, followed by CYP1B1, are the most efficient enzymes oxidizing ellipticine to 7-hydroxy- and 9-hydroxyellipticine (Figure 3) [22]. Thus, CYP1A are the most important enzymes detoxifying ellipticine. The activation metabolite, 13-hydroxyellipticine, forming the ellipticine-DNA adduct 1 (Figure 2C), is generated predominantly by CYP3A4. Oxidation of ellipticine to another activation metabolite, 12-hydroxyellipticine, generating DNA adduct 2 (Figure 2D) is also catalyzed by CYP3A4, but more efficiently by CYP2C19. The N^2-oxide of ellipticine is generated beside CYP3A4 mainly by CYP2D6 [22,25,26,30]. These results demonstrate that CYP3A4 is the most effective enzyme that forms both ellipticine-DNA adducts 1 and 2 (see adducts 1 and 2 in Figures 2 and 3), while adduct 2 is also generated by CYP2C19 and 2D6. Moreover, orthologous CYP enzymes of rats and mice catalyze formation of these metabolites and DNA adducts [1-4,27,29]. This indicates that these animals might be suitable models mimicking the fate of ellipticine in human.

Recently it was also found that levels of the DNA adduct formed by 13-hydroxyellipticine increased if this ellipticine metabolite was conjugated with sulfate or acetate by human sulfotransferases 1A1, 1A2, 1A3 and 2A1, or *N,O*-acetyltransferases 1 and 2 (Figure 3) [25,46].

Beside CYP enzymes, peroxidases are capable of oxidizing ellipticine. *In vitro*, human myeloperoxidase, bovine lactoperoxidase, ovine cyclooxygenase (COX)-1, human COX-2 and plant horseradish peroxidase are able to bio-activate ellipticine to form up to four DNA adducts [23]. Interestingly, even though mechanisms of oxidation of a variety of substrates including ellipticine by peroxidases and CYPs are different, two of the DNA adducts formed during oxidation of ellipticine by peroxidases are identical to those produced by 13-hydroxy- and 12-hydroxyellipticine generated by CYPs [23].

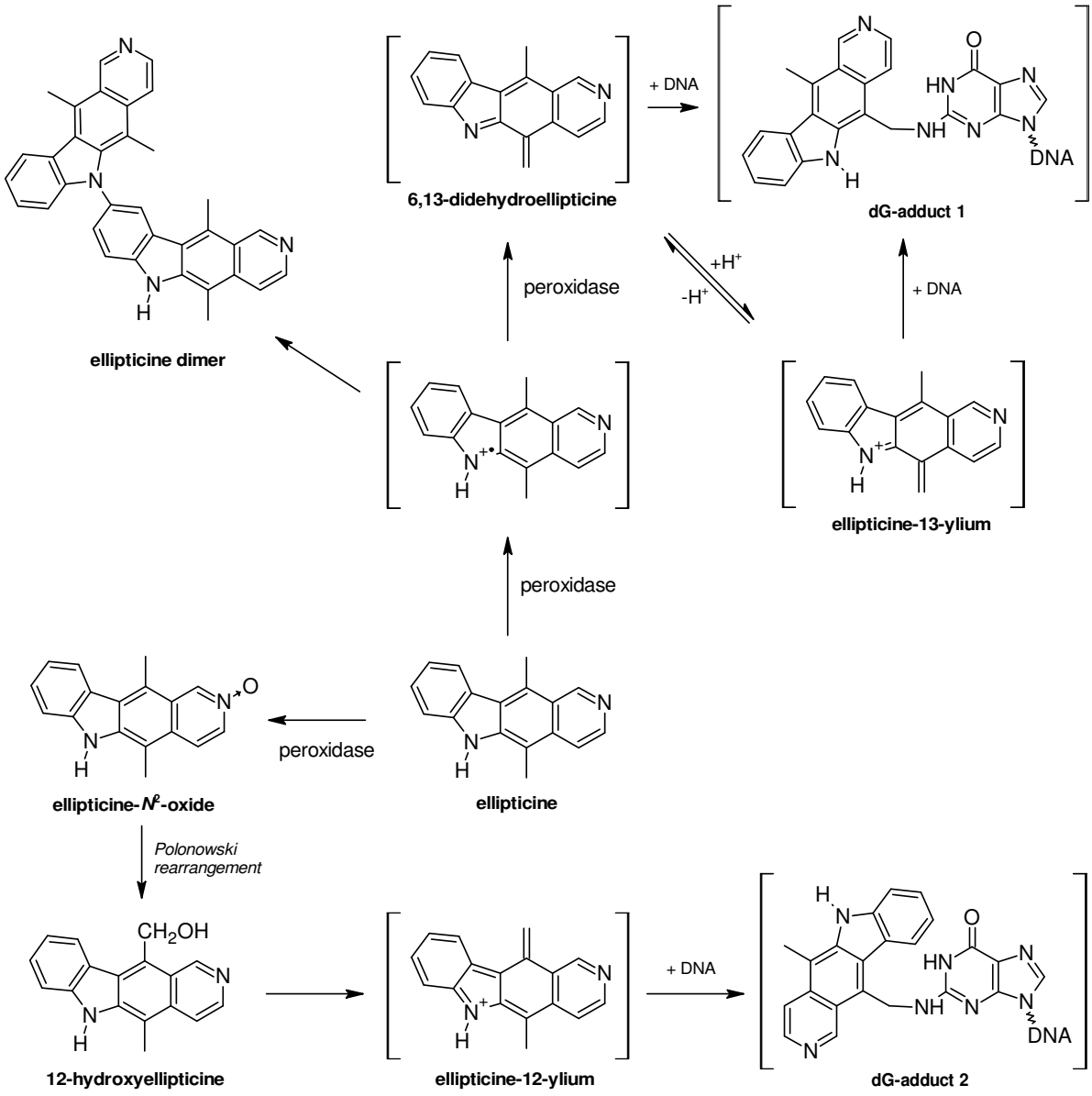
Figure 3. Ellipticine metabolism by CYPs showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and/or not yet structurally characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the figure were identified in our previous studies [22,25,26,29,30]. Rea 1, 2 and 3 are reactions leading to ellipticine-13-ylium from 13-hydroxyellipticine, 13-hydroxyellipticine sulfate and 13-hydroxyellipticine acetate, respectively.



Peroxidases oxidize ellipticine by a one-electron (radical) mechanism to two metabolites, the major metabolite is an ellipticine dimer between the N6 of one ellipticine molecule and C9 of another ellipticine molecule, and the second, minor one is ellipticine N^2-oxide (Figure 4) [23]. The ellipticine dimer is not responsible for ellipticine-DNA adduct formation, but is formed when no other nucleophiles like DNA or proteins are present to intercept the reactive intermediates of ellipticine [23,47]. Hence, adduct formation is preferred to generation of dimer under physiological conditions.

Figure 4. Metabolism of ellipticine by peroxidases showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected

195 under the experimental conditions and are the electrophilic metabolites postulated as ultimate
196 arylating species or the postulated N^2-deoxyguanosine adducts (adapted from reference [23]).



197
198

199

200 Because DNA adduct 1 formed by 13-hydroxyellipticine is identical to the DNA adduct generated
201 during ellipticine oxidation by peroxidases, 6,13-didehydroellipticine (the ellipticine methylene-imine)
202 (Figure 4), was postulated to be the metabolite responsible for its generation. This compound should
203 form ellipticine-13-ylium, the identical carbonium-cation as 13-hydroxyellipticine (Figure 4). Michael-
204 type addition of nucleophiles to the vinylogous imine of 6,13-didehydroellipticine then yields the same
205 DNA adduct as the adduct generated by ellipticine-13-ylium [48]. The ellipticine-DNA adduct 2
206 mediated by peroxidase is identical to that formed by 12-hydroxyelliptine. Because this metabolite is
207 formed not only by ellipticine oxidation with CYPs, but also by the Polonowski rearrangement from
208 ellipticne N^2-oxide that is generated by peroxidases [23,45], the mechanism of its formation is easily
209 explained. The ellipticine-12-ylium carbo-cation formed by dissociation of 12-hydroxyellipticine reacts

with deoxyguanosine in DNA to form the DNA adduct 2 (Figure 4) [23]. The other two DNA adducts, which are formed by the peroxidase-catalyzed activation of ellipticine (spots 6 and 7 in Figure 2) [23], are also observed during oxidation of ellipticine by microsomes isolated from livers of humans [22,49], rats [1,29,49], rabbits [1,29], and mice [39,50], and in several organs of mice (Figure 2E,F) and rats (Figure 2G) exposed to ellipticine [24,28,39]. In contrast, the other minor ellipticine-DNA adducts formed *in vivo* (see spots 3-5 in Figure 2) are not produced by ellipticine oxidation with peroxidase [23]. The structures of these minor ellipticine-DNA adducts (spots 3-7 in Figure 2) have not been identified as yet.

4. Oxidation of ellipticine by CYP enzymes to activation and detoxification metabolites *in vivo*

In order to extrapolate from the *in-vitro* data to the *in-vivo* situation, additional factors have to be considered such as route-of-administration, absorption, renal clearance, and tissue-specific expression of enzymes metabolizing ellipticine. To identify CYP enzymes responsible for activation of ellipticine *in vivo*, several animal models were used: (*i*) the wild-type (WT) and the Hepatic P450 Reductase Null (HRN) mice, (*ii*) the same mouse models, but in which expression of enzymes of the mixed-function oxidase system was induced by benzo[a]pyrene (BaP), and (*iii*) the Wistar rats. These studies demonstrated that utilizing knockout animals in combination with animal models, where expression of these enzymes was modulated by their inducers, is crucial for determining the fate of ellipticine and its DNA-damaging effects *in vivo*. In addition, the results found in experiments utilizing these models revealed a paradox: namely, the finding that CYP1A enzymes appear to be more important for activation of ellipticine *in vivo*, despite being involved in its metabolic detoxification *in vitro*.

4.1. Utilization of WT and HRN mice to identify enzymes metabolizing ellipticine in vivo

In HRN mice, mouse POR, the most important electron donor to mouse CYPs, is deleted specifically in hepatocytes. This model was developed by Henderson et al. [51] to evaluate the role of both hepatic POR and CYPs in xenobiotic metabolism. Deletion of this enzyme results not only in the loss of essentially all hepatic CYP function, but also in the lack of direct reduction of xenobiotics by POR, an additional property of this enzyme. This mouse model has been used successfully to investigate the role of hepatic *versus* extra-hepatic CYP-catalyzed metabolism and the disposition of several xenobiotics such as carcinogens and drugs including ellipticine [39,52-58].

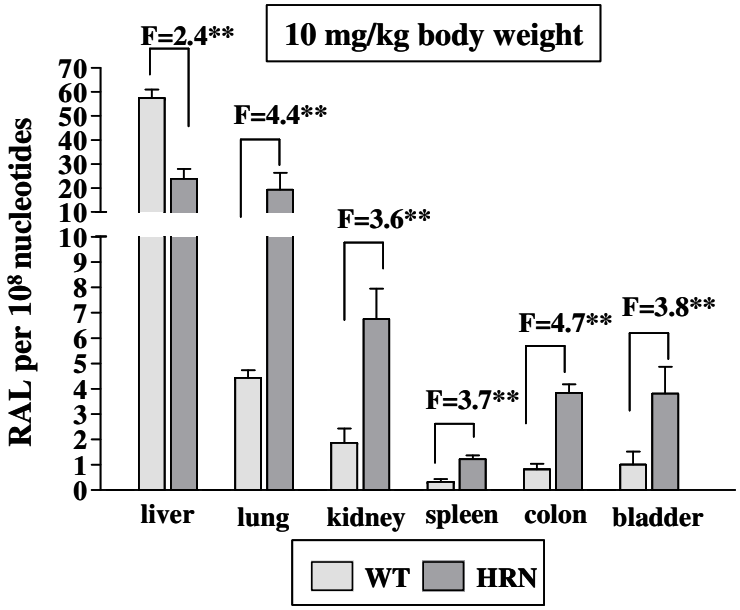
Using WT and HRN mouse lines, hepatic CYPs were demonstrated to be important in ellipticine-derived DNA adduct formation also *in vivo*. In both mouse lines treated with ellipticine, up to seven ellipticine-specific DNA adducts were observed in liver, lung, kidney, spleen, colon and bladder (see Figure 2E,F for liver of WT and HRN mice). Adduct spots 1 and 2 derived from 13-hydroxy- and 12-hydroxyellipticine, respectively, and deoxyguanosine residues in DNA (Figures 2 and 3) were the predominant adducts in all mouse tissues examined.

The finding that ellipticine-DNA adducts are formed in all organs tested in used animal models suggest that ellipticine or its metabolites are distributed *via* the blood stream to different organs and that these tissues have the metabolic capacity to oxidatively activate ellipticine. As found by Chadwick and co-workers [43,44] ellipticine is very rapidly distributed from the blood, and its excretion is essentially complete by 24 hours in several species including mice, rats, dogs, and monkeys. The rate

of ellipticine elimination from blood was found to reflect the rate of metabolism of this drug [43]. The main organ responsible for its biotransformation was found to be the liver, forming predominantly 9-hydroxyellipticine, which is excreted mainly in bile as its glucuronide or sulfate conjugate [43,44]. Other *in-vivo* pathways involving hydroxylation at as yet unknown positions have also been found [43,44]. As mentioned above, in *in-vitro* experiments, ellipticine is metabolized by CYP-mediated reactions by hepatic microsomes of a variety of organisms, including humans, rats, rabbits and mice [22,30,39,40,49,50] to several hydroxylated derivatives, with 9-hydroxy-, 12-hydroxy- and 13-hydroxyellipticine being the major metabolites in most species. However, because 13-hydroxy- and 12-hydroxyellipticine are reactive and have been found to form the two major ellipticine-DNA adducts [22,23,30,38], they will not be easily detectable *in vivo*. In addition, radioactively labelled ellipticine was found to be deposited in a number of organs with the highest levels in the liver, followed by kidney, lung, intestine and spleen, and was located primarily in the nuclear fraction [43]. One of the explanation for this may be the ellipticine-DNA binding that we found in these tissues [23,24,39].

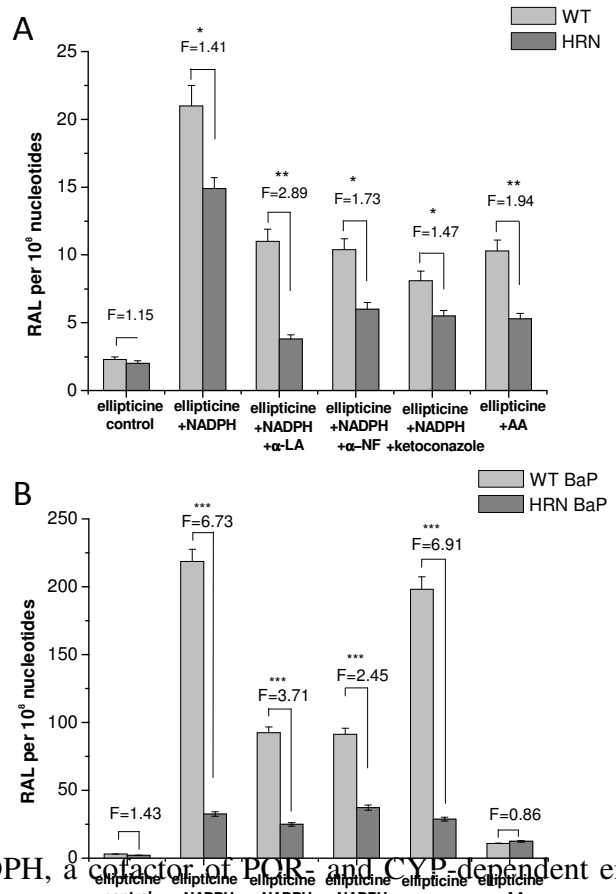
The levels of ellipticine-DNA adducts in the livers of HRN mice were lower (by up to 65%) than in the livers of WT mice, demonstrating that CYP enzyme activity is important for the oxidative activation of ellipticine to metabolites generating these adducts. Whereas hepatic CYP-mediated ellipticine DNA binding was reduced in HRN mice, DNA binding in extrahepatic organs was increased (Figure 5). Up to 4.7-fold higher levels of DNA adducts were found in extra-hepatic organs of HRN mice than of WT mice (Figure 5), suggesting that these tissues have the metabolic capacity to oxidize ellipticine and, more importantly, that the same reactive species forming DNA adducts are produced in the liver, probably by both CYP-catalyzed ellipticine activation and also independently of activation by these enzymes.

Figure 5. Total levels of ellipticine-DNA adducts determined and quantified by ^{32}P-postlabelling analysis of DNA isolated from organs of HRN and WT mice treated *i.p.* with (10 mg ellipticine/kg body weight. F = fold higher and /or lower DNA adducts in HRN than WT mice. *Columns*, mean; *bars*, SD (*n*=3); each DNA sample was analysed twice. **P<0.05*, ***P<0.01*, ****P<0.001*. RAL, relative adduct labelling. ND = not detected.



Experiments utilizing the *ex-vivo* incubations of ellipticine with hepatic microsomes of WT and HRN mice and those of these mice exposed to BaP, as well as employing inhibitors of the most important CYP enzymes catalyzing detoxification and activation of ellipticine *in vitro*, CYP1A and 3A, respectively, help to resolve which of these CYPs play a role in the used mouse models. As it was expected, treatment of WT and HRN mice with BaP led to a significant increase in expression of CYP1A, predominantly of CYP1A1 in liver (up to 175-fold), both at the transcriptional and translation levels [50,54]. This carcinogen increased also the expression of POR protein and its enzymatic activity in livers of these mouse models, but to a much lower extent, up to 2.9-fold. More interestingly, exposure of WT and HRN mice to BaP was also found to results in an increase in expression of another protein of the microsomal mixed-function-oxidase enzymatic system, cytochrome b_5, in livers of these mice [55].

Figure 6. DNA adduct formation by ellipticine activated with microsomes isolated from livers of untreated Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice **(A)** and from those treated with BaP **(B)** as determined by thin layer chromatography (TLC) ^{32}P-postlabeling. F = fold higher DNA adducts levels in microsomes from WT mice compared to HRN mice. *Columns:* Mean RAL (relative adduct labeling) ± standard deviations (S.D.) shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in vitro* incubations). Values significantly different from HRN mice:*$P<0.05$, **$P<0.01$, ***$P<0.001$. Control = without cofactor; AA = arachidonic acid; α-NF = α-naphthoflavone; α-LA = α-lipoic acid. ND = not detected.

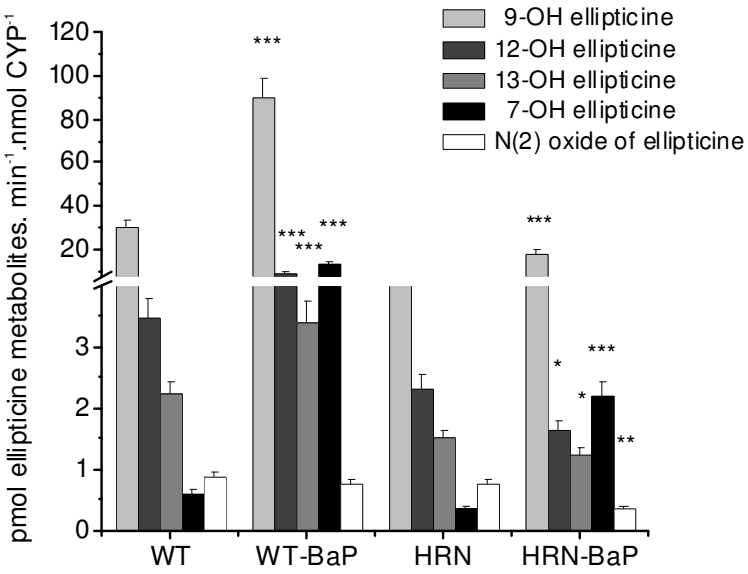


In the presence of NADPH, a cofactor of POR- and CYP-dependent enzyme systems, the *ex-vivo* incubations with ellipticine, DNA and hepatic microsomes of untreated (control) WT and HRN mice

328 and mice treated with BaP led to activation of this drug to ellipticine-derived DNA adducts (Figures 2
329 and 6), confirming a role of CYPs in ellipticine activation. Arachidonic acid, a cofactor for COX-
330 dependent oxidation [23,53,59-61], mediated formation of ellipticine-DNA adducts 1 and 2 in hepatic
331 microsomes of all mice used, too. This suggests that COX also activate ellipticine in mouse liver
332 [39,50]. But arachidonic acid as a cofactor of this enzyme was much less effective than a cofactor of
333 POR and CYP, NADPH (Figure 6).
334 Surprisingly, levels of ellipticine-derived DNA adducts formed in the *ex-vivo* incubations in the
335 presence of NADPH were only 1.4-fold lower than amounts formed by hepatic microsomes from WT
336 mice (Figure 6), even though POR expression in livers of HRN mice was two orders of magnitude
337 lower. This finding indicates that ellipticine activation should, at least partially, be catalyzed also by
338 enzymes, whose activities are not dependent on POR [39]. Beside peroxidases that were found to
339 activate ellipticine [23], the CYP2S1 enzyme, which is abundantly expressed in several tissues [62-65]
340 might be such an enzyme. Namely, it was shown that it catalyzes the oxidation of compounds having
341 polycyclic aromatic structures similar to ellipticine without participation of POR [64,65]. Whereas a
342 role of a COX peroxidase in hepatic microsomes of WT and HRN mice was proved in the study with
343 these animal models (see Figure 6) [39,50], the participation of CYP2S1 in ellipticine activation awaits
344 further examination. Therefore, the human recombinant CYP2S1 enzyme heterogously expressed in
345 *Escherichia coli* was prepared in our laboratory [66] and will be utilized to investigate efficiency of
346 this CYP in ellipticine oxidation in an additional study.
347 At least two adducts (spots 1 and 2 in Figure 2A,B), which were identical to those generated *in vivo*
348 in mice treated with ellipticine (Figure 2E,F) were formed by mouse hepatic microsomes. Furthermore,
349 ellipticine-derived DNA adduct, spot A, was found as a minor adduct (Figure 2A), predominantly in
350 microsomes isolated from HRN mice [39]. In incubations containing hepatic microsomes of WT and
351 HRN mice treated with BaP, an additional adduct spot, corresponding to the 10-(deoxyguanosin-*N*2-
352 yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG-N^2-BPDE) adduct that is the major
353 product of reaction of BaP metabolite BaP-7,8-dihydrodiol-9,10-epoxide with DNA *in vitro* and *in vivo*
354 [54,67] was also detected (Figure 2B). This finding indicates that residual BaP is present in
355 microsomes isolated from livers of WT and HRN mice treated with BaP, and is activated by CYP1A1
356 in combination with microsomal epoxide hydrolase to form this adduct.
357 Ketoconazole, a selective inhibitor of CYP3A enzymes [68,69], inhibited formation of ellipticine-
358 DNA adducts in hepatic microsomes of untreated (control) WT and HRN mice, by ~60% (Figure 6A),
359 confirming a role of CYP3A in ellipticine activation in mouse liver. However, the effect of this
360 inhibitor was much lower in hepatic microsomes of BaP-treated WT and HRN mice, only by ~10%
361 (Figure 6B). Hence, a contribution of CYP3A to formation ellipticine-derived DNA adducts in livers
362 was decreased by induction of CYP1A1 with BaP. These results point additionally to CYP3A enzymes
363 as having a role in ellipticine-DNA adduct formation in mouse livers, but their contributions to this
364 process was decreased by induction of CYP1A with BaP. Surprisingly, an increased level of CYP1A
365 by its induction with BaP, the enzymes that mainly detoxify ellipticine *in vitro*, led to an increase in
366 amounts of ellipticine-DNA adducts formed (Figure 6), predominantly of adduct 1 [50]. Moreover, a
367 selective inhibitor of CYP1A activities, α-naphthoflavone (α-NF) [68], decreased formation of
368 ellipticine-DNA adducts. Both these findings prove that mouse hepatic CYP1A are required for
369 ellipticine activation (Figure 6). Induction of CYP1A in HRN mice by BaP also resulted in an increase

in levels of ellipticine-DNA aducts, however, in hepatic microsomes of these HRN mice, α-NF caused an increase rather than a decrease in formation of ellipticine-DNA adducts (Figure 6). Therefore, here the amounts of CYP1A enzymes induced by BaP seem to increase ellipticine detoxification (see also below).

Figure 7. Levels of ellipticine metabolites formed by hepatic microsomes (0.2 mg protein) of Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice from 10 μM ellipticine and by hepatic microsomes of HRN and WT mice treated intraperitoneally (*i.p.*) with 5 × 125 mg of benzo[a]pyrene (BaP) per kg of body weight. Levels of ellipticine metabolites were determined by high performance liquid chromatography (HPLC) [22,49] and are averages ± standard deviations of triplicate incubations. Values significantly different from untreated mice: **P<0.05, **P<0.01, ***P<0.001*



Ellipticine metabolites formed in hepatic microsomes from all mouse lines used in previous studies [39,50] were analogous; 9-hydroxy-, 12-hydroxy-, 13-hydroxy, 7-hydroxyellipticine and N^2-oxide of ellipticine were formed (Figure 7). However, the patterns of individual metabolites in WT and HRN mice, either control (untreated) or treated with BaP, were different. In the case of untreated mice, 9-hydroxyellipticine levels were only one sixth, while the amounts of 13-hydroxy- and 12-hydroxyellipticine, were about one half in incubations with HRN microsomes compared with the levels in incubations with WT microsomes. Exposure of both WT and HRN mice to BaP inducing CYP1A resulted, as it was expected, in an increase in formation of 9-hydroxy- and 7-hydroxyellipticine, the metabolites that are considered to be detoxification products (Figure 7). This result is consistent with the former studies, where CYP1A1 and 1A2 were the major enzymes forming these metabolites [22,26,31]. However, treatment of WT mice with BaP also resulted in up to 2.5-fold increased levels of 13-hydroxy- and 12-hydroxyellipticine (Figure 7), the metabolites that were found to be formed *in vitro* mainly by CYP3A, and much less efficiently by CYP1A1 [22,30]. Hence not only CYP3A, but also CYP1A expressed in mouse liver are important to form 13-hydroxy- and 12-hydroxyellipticine generating ellipticine-DNA adducts. These findings show that in contrast to the pure *in vitro* CYP systems (CYPs reconstituted with POR), CYP1A enzymes are responsible for ellipticine activation to

form DNA adducts in mouse liver [39,50]. These discrepancies were explained in further studies (see bellow).

4.2. Utilization of Wistar rats to identify enzymes metabolizing ellipticine in vivo

In order to identify CYP enzymes responsible for activation and detoxification of ellipticine *in vivo* further, Wistar rats were used as another animal model. Also in this animal model, when treated with ellipticine, ellipticine-derived DNA adducts were generated in DNA of several healthy organs (liver, kidney, lung, spleen, breast, heart and brain) (see Figure 2G for rat liver) [3,24,28] and in DNA of breast adenocarcinoma [3]. The levels of ellipticine-derived DNA adducts generated in breast adenocarcinoma were almost 2-fold higher than in normal healthy breast tissue. This finding indicates that CYP enzymes activating ellipticine should be expressed at higher levels in this breast adenocarcinoma such as a typical CYP expressed in breast cancer, CYP1B1 [32], than in peritumoral tissues. Indeed, such results were found by us [32] and by other authors previously [70-72].

Also in rats, formation of ellipticine-DNA adduct 1 is dependent not only on levels of CYP3A, but also on those of CYP1A1. The levels of ellipticine DNA adduct 1 in analyzed organs correlated not only with expression levels of CYP3A, but also with that of CYP1A1 in these organs, which does not corresponds to situation *in vitro*. As outlined above, in the *in-vitro* systems, CYP3A is mainly responsible for formation of ellipticine-DNA adducts and CYP1A predominantly oxidizes ellipticine to its detoxification metabolites [3,4,27]. The *in-vitro* studies investigating the effect of cytochrome b_5 on the metabolism of ellipticine explained the discrepancies between CYP enzymes oxidizing ellipticine *in vitro* and *in vivo*, and found a crucial role of this protein in contribution of individual CYP enzymes to ellipticine activation and detoxification. Moreover, increased protein expression of cytochrome b_5 in liver of rats treated with ellipticine [26,42] suggests that cytochrome b_5 may modulate the CYP-mediated bioactivation and detoxification of ellipticine in this animal model *in vivo*.

Cytochrome b_5 is an important component of the microsomal mixed-function-oxidase system and can influence the metabolism of xenobiotics [58,67,73-78]. For more than four decades, the role of cytochrome b_5 in CYP catalysis has been controversial, and based entirely on *in vitro* data, which showed that cytochrome b_5 could inhibit or stimulate CYP activity dependent on a number of variables including CYP isoenzyme, substrate and cytochrome b_5 concentration [73-78]. In order to investigate the role of cytochrome b_5 in ellipticine metabolism we conducted some *in vitro* experiments using human liver microsomes, hepatic microsomes from control and ellipticine-pretreated rats and reconstituted systems with CYP1A1, CYP1A2, CYP3A4, POR, and cytochrome b_5 in different ratios [25,26,30,40,42,80]. We have found that cytochrome b_5 alters the ratio of ellipticine metabolites generated by CYP1A1, 1A2, and 3A4. Whereas the amounts of the detoxication ellipticine metabolites (7-hydroxy- and 9-hydroxyellipticine) are either decreased (CYP1A1/2) or not changed (CYP3A4) with cytochrome b_5 added to the reconstituted system, the amounts of the activation metabolites, 12-hydroxy- and 13-hydroxyellipticine, increased considerably. The change in the amounts of metabolites found in these studies resulted in higher ellipticine-DNA adduct levels [25,26,30]. In these enzymatic systems the ratios of the various partners of CYP are artificially generated, therefore a more physiological model to identify human enzymes responsible for ellipticine activation was used, namely, human hepatic microsomes [22,30]. These microsomal fractions contain a mixture of human CYPs,

POR, cytochrome b_5 and its reductase (NADH:cytochrome b_5 reductase). Thus, they comprise the essential components of the enzymatic system metabolizing drugs, mimicking well a situation in human liver, where a majority of drug metabolism occurs [67,79]. Human hepatic microsomes oxidize ellipticine mainly to 13-hydroxy- and 12-hydroxyellipticine, whereas 7-hydroxy-, 9-hydroxyellipticine and ellipticine N^2-oxide are generated at more than 10-fold lover amounts (see Table 2 in [30]). Similar results were found in hepatic microsomes of rats [49]. As a consequence, high levels of both major ellipticine-DNA adducts are formed when DNA is added to the microsomal incubations. The amounts formed correlated with the activity of the major CYPs found previously to form the metabolites generating these DNA adducts (see above) [22,30].

All these results explained the reasons why the CYP1A enzymes are more important in ellipticine activation *in vivo*; their activity is modulated by cytochrome b_5. These results also demonstrated that not only the expression levels of CYP1A and 3A in several organisms including human, but also the amounts of expressed cytochrome b_5 dictate the oxidative activation and detoxification of ellipticine *in vivo*. Moreover, because ellipticine itself is capable of inducing expression of CYP1A (predominantly CYP1A1), POR, and cytochrome b_5 [42,80], it increases its own metabolism leading predominantly to activation of this drug to reactive species forming DNA adducts [25,26], thereby modulating its own pharmacological potential.

The ellipticine-derived DNA adducts formed by enzymatic activation of ellipticine *in vitro* and *in vivo* were also found in tumor cells, in which CYP enzymes are expressed. The adducts were found in human breast adenocarcinoma MCF-7 cells [32], neuroblastoma IMR-32, UKF-NB-3, and UKF-NB-4 cells [34,35], glioblastoma U87MG cells [37], and BHT-101, B-CPAP and 8505-C thyroid cancer cells [36] exposed to ellipticine. Cytotoxicity of ellipticine corresponds to amounts of ellipticine-DNA adducts formed in the tested cancer cells and depends on expression levels of CYP enzymes metabolizing ellipticine (CYP1A1, 1B1, and 3A4) and/or cytochrome b_5 in these cells [27,32,34-37]. The high expression levels of cytochrome b_5 together with those of CYP1A1 and 3A4 dictate the DNA adduct formation by and cytotoxicity of ellipticine also in most of these cancer cells, predominantly in neuroblastoma UKF-NB-4, glioblastoma U87MG and thyroid cancer cell lines [11,27,32,34-36]. These findings again demonstrate a role not only of the expression of CYPs, but also of cytochrome b_5, because it dictates the pharmacological effects of ellipticine.

Concerning formation of ellipticine-derived DNA adducts in the healthy organs of experimental models *in vivo* [24,28,39], it should be emphasized that expression levels of CYP enzymes activating ellipticine in these tissues might determine also genotoxic side effects of ellipticine in these tissues. Indeed, several studies have found a positive correlation between DNA adduct levels of carcinogens or genotoxic agents, their persistence and their mutagenicity in the target organ and/or tumor formation [81-85]. Therefore, to better understand the role of ellipticine-DNA adducts in such genotoxic side effects of ellipticine in the cancer treatment, we have analyzed the dose dependence and the persistence of ellipticine-DNA adducts in non-target tissues (liver, lung, kidney, spleen, heart, and brain) of rats, the animal model mimicking the bioactivation of ellipticine in human, treated with ellipticine [24]. Only very low levels of the adducts and only in some of tissues analyzed in such a study were retained in DNA of these non-target tissues. In addition, not all of the ellipticine-DNA adducts persist in the tissues analyzed in the study (only adducts 1, 2, 4, and 5) [24]. This finding demonstrates that healthy tissues of rats treated with ellipticine posses effective repairing systems to remove certain lesions and

suggests a relatively low impact of the genotoxic side effects of ellipticine during the cancer treatment in human.

5. Conclusions

The data summarized in this review demonstrate that a DNA-damaging anticancer alkaloid ellipticine might be considered as a prodrug, whose major mechanism of action is mediated by the enzyme-catalyzed activation leading to formation of covalent DNA adducts in target tissues. These ellipticine-DNA adducts are formed in both healthy and tumor tissues, but they do not persist in healthy tissues. The data show that cytotoxic effects of ellipticine in tumor tissues (cells) are dictated by (i) levels of CYP expression (and/or peroxidase expression), (ii) levels of cytochrome b_5 expression, and (iii) its own potency to induce CYP1A1, CYP3A and cytochrome b_5 in tumor tissues (cells). The results found also demonstrate that animal models, where levels of biotransformation enzymes are regulated both by their knockout or induction are appropriate tools to identify enzymes responsible for ellipticine metabolic activation and detoxification. They also demonstrate that extrapolation from *in vitro* data to situation *in vivo* is not always possible, confirming the need for these animal models.

Even though the role of cytochrome b_5 in modulation of ellipticine metabolism *in vitro* was clearly proved, its effect *in vivo* is still quite enigmatic. Two mouse lines such as a line that has been generated with a conditional hepatic deletion of cytochrome b_5 (HBN, Hepatic cytochrome b_5 Null) [86] and a double conditional mutant, HBRN (Hepatic cytochrome b_5 /P450 Reductase Null), in which both enzymes are deleted specifically in the liver [87], recently developed in the laboratory of Wolf and coworkers [86,87] can provide a study to resolve the role of cytochrome b_5 in CYP-mediated metabolism of ellipticine *in vivo*.

The results summarized in this review form the basis to further predict the susceptibility of human cancers to ellipticine and suggest this alkaloid for treatment in combination with CYP gene transfer (CYP-gene-directed enzyme-prodrug therapy) [88,89], which has the potential to provide efficient activation of ellipticine in target tumor tissue, thereby increasing the anticancer potential of this prodrug. Furthermore, two of the ellipticine metabolites formed by oxidation with CYPs in combination with cytochrome b_5, 13-hydroxy- and 12-hydroxyellipticine, are reactive enough to decompose spontaneously to the carbenium ions forming DNA adducts that are predominantly responsible for killing cancer cells. Both these ellipticine metabolites are, therefore, excellent candidates for tumor cell-specific targeting to these cancers that include the use of systemic delivery of these metabolites. Therefore, we suggest these two ellipticine derivatives for potential clinical usage. Further, preparation of appropriate derivatives including their encapsulated forms in nanocarriers that would deliver them to cancer cells is one of the major challenges in research of our laboratory to prepare suitable ellipticine derivatives for clinical usage.

Acknowledgments

This work was supported by the Grant Agency of the Czech Republic (grant 14-18344S in panel P301) and Charles University (UNCE204025/2012). Work at King's College London is supported by

Cancer Research UK and the Wellcome Trust. VMA is a member of the Wellcome funded COMSIG (Causes Of Mutational SIGnatures) consortium.

Author Contributions

Conception and design: Stiborová Marie, Černá Věra, Moserová Michaela, Mrízová Iveta, Frei Eva
Analysis and interpretation of the data: Stiborová Marie, Arlt Volker M., Frei Eva
Drafting of the article: Stiborová Marie, Frei Eva
Critical revision of the article for important intellectual content: Stiborová Marie, Arlt Volker M., Frei Eva,

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Stiborova, M.; Bieler, C.A.; Wiessler, M.; Frei, E. The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. *Biochem. Pharmacol.* **2001**, *62*, 1675-1684.
2. Stiborova, M.; Rupertova, M.; Schmeiser, H.H.; Frei, E. Molecular mechanisms of antineoplastic action of an anticancer drug ellipticine. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.* **2006**, *150*, 13-23.
3. Stiborova, M.; Rupertova, M.; Frei, E. Cytochrome P450- and peroxidase-mediated oxidation of anticancer alkaloid ellipticine dictates its anti-tumor efficiency. *Biochim. Biophys. Acta* **2011**, *1814*, 175-185.
4. Kizek, R.; Adam, V.; Hrabeta, J.; Eckschlager, T.; Smutny, S.; Burda, J.V.; Frei, E.; Stiborova, M. Anthracyclines and ellipticines as DNA-damaging anticancer drugs: recent advances. *Pharmacol. Ther.* **2012**, *133*, 26-39.
5. Auclair, C. Multimodal action of antitumor agents on DNA: the ellipticine series. *Arch. Biochem. Biophys.* **1987**, *259*, 1-14.
6. Garbett, N.C.; Graves, D.E. Extending nature's leads: the anticancer agent ellipticine. *Curr. Med. Chem. Anti-Cancer Agents* **2004**, *4*, 149-172.
7. Ohashi, M.; Sugikawa, E.; Nakanishi, N. Inhibition of p53 protein phosphorylation by 9-hydroxyellipticine: A possible anticancer mechanism. *Jpn. J. Cancer Res.* **1995**, *86*, 819-829.
8. Shi, L.M.; Myers, T.G.; Fan, Y.; O'Conno,r P.M.; Paull, K.D.; Friend, S.H.; Weinstein, J.N. Mining the National Cancer Institute Anticancer Drug Discovery Database: cluster analysis of ellipticine analogs with p53-inverse and central nervous system-selective patterns of activity. *Mol. Pharmacol.* **1998**, *53*, 241-251.
9. Kuo, P.L.; Hsu, Y.L.; Chang, C.H.; Lin, C.C. The mechanism of ellipticine-induced apoptosis and cell cycle arrest in human breast MCF-7 cancer cells. *Cancer Lett.* **2005**, *223*, 293-301.

10. Kuo, P.L.; Hsu, Y.L.; Chang, C.H.; Lin, C.C. The antiproliferative inhibition of ellipticine in human breast mda-mb-231 cancer cells is through cell cycle arrest and apoptosis induction. *Anti-Cancer Drugs* **2005**, *16*, 789-795.
11. Martinkova, E.; Maglott, A.; Leger, D.Y.; Bonnet, D.; Stiborova, M.; Takeda, K.; Martin, S.; Dontenwill, M. α5β1 integrin antagonists reduce chemotherapy-induced premature senescence and facilitate apoptosis in human glioblastoma cells. *Int. J. Cancer*. **2010**, *127*, 1240-1248.
12. Sugikawa, E.; Hosoi, T.; Yazaki, N.; Gamanuma, N.; Nakanishi, N.; Ohashi, M. Mutant p53 mediated induction of cell cycle arrest and apoptosis at G1 phase by 9-hydroxyellipticine. *Anticancer Res.* **1999**, *19*, 3099-3108.
13. Peng, Y.; Li, C.; Chen, L.; Sebti, S.; Chen, J. Rescue of mutant p53 transcription function by ellipticine. *Oncogene* **2003**, *22*, 4478-4487.
14. Fritsche, M.; Haessler, C.; Brandner, G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* **1993**, *8*, 307–318.
15. Xu, G.W.; Mawji, I.A.; Macrae, C.J.; Koch, C.A.; Datti, A.; Wrana, J.L.; Dennis, J.W.; Schimmer, A.D. A high-content chemical screen identifies ellipticine as a modulator of p53 nuclear localization. *Apoptosis* **2008**, *13*, 413-422.
16. Kohn, K.W.; Waring, M.J.; Glaubiger, D.; Friedman, C.A. Intercalative binding of ellipticine to DNA. *Cancer Res.* **1975**, *35*, 71–6.
17. Patel, N.; Bergman, J.; Gräslund, A. ^{1}H-NMR studies of the interaction between a self-complementary deoxyoligonucleotide duplex and indolo[2,3-b]quinoxaline derivatives active against herpes virus. *Eur. J. Biochem.* **1991,** *197*, 597-604.
18. Chu, Y.; Hsu, M.T. Ellipticine increases the superhelical density of intracellular SV40 DNA by intercalation. *Nucleic Acids Res* ,**1992**, *20*, 4033-4038.
19. Singh, M.P.; Hill, G.C.; Peoch, D.; Rayner, B.; Inabach, J.L.; Lown, J.W. High-field NMR and restrained molecular modeling studies on a DNA heteroduplex containing a modified apurinic abasic site in the form of covalently linked 9-aminoellipticine. *Biochemistry* **1994**, *33*, 10271-10285.
20. Belehradek, J. Jr; Fermandjian, S. DNA-drug recognition and effects on topoisomerase II-mediated cytotoxicity. A three-mode binding model for ellipticine derivatives. *J. Biol. Chem.* **1991**, *266*, 1820-1829.
21. Froelich-Ammon, S.J.; Patchan, M.W.; Osheroff, N.; Thompson R.B. Topoisomerase II binds to ellipticine in the absence or presence of DNA. Characterization of enzyme-drug interactions by fluorescence spectroscopy. *J. Biol. Chem.* **1995**, *270*, 14998-5004.
22. Stiborová, M.; Sejbal, J.; Borek-Dohalská, L.; Aimová, D.; Poljaková, J.; Forsterová, K.; Rupertová, M.; Wiesner, J.; Hudecek, J.; Wiessler, M.; Frei, E. The anticancer drug ellipticine forms covalent DNA adducts, mediated by human cytochromes P450, through metabolism to 13-hydroxyellipticine and ellipticine N^2-oxide. *Cancer Res.* **2004**, *64*, 8374-8380.
23. Stiborová, M.; Poljaková, J.; Ryslavá, H.; Dracínský, M.; Eckschlager, T.; Frei, E. Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming

deoxyguanosine adducts in DNA identical to those found *in vivo* and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. *Int. J. Cancer* **2007**, *120*, 243-251.

24. Stiborová, M.; Rupertová, M.; Aimová, D.; Ryslavá, H.; Frei, E. Formation and persistence of DNA adducts of anticancer drug ellipticine in rats. *Toxicology* **2007**, *236*, 50-60.
25. Stiborová, M.; Indra, R.; Moserová, M.; Cerná, V.; Rupertová, M.; Martínek, V.; Eckschlager, T.; Kizek, R.; Frei, E. Cytochrome b_5 increases cytochrome P450 3A4-mediated activation of anticancer drug ellipticine to 13-hydroxyellipticine whose covalent binding to DNA is elevated by sulfotransferases and *N,O*-acetyltransferases. *Chem. Res. Toxicol.* **2012**, *25*, 1075-1085.
26. Kotrbová, V.; Mrázová, B.; Moserová, M.; Martínek, V.; Hodek, P.; Hudeček, J.; Frei, E.; Stiborová, M. Cytochrome b_5 shifts oxidation of the anticancer drug ellipticine by cytochromes P450 1A1 and 1A2 from its detoxication to activation, thereby modulating its pharmacological efficacy. *Biochem. Pharmacol.* **2011**, *82*, 669-680.
27. Stiborova, M., Frei, E. Ellipticines as DNA-targeted chemotherapeutics. *Current Med. Chem.* **2014,** *21*, 575-591.
28. Stiborová, M.; Breuer, A.; Aimová, D.; Stiborová-Rupertová, M.; Wiessler, M.; Frei, E. DNA adduct formation by the anticancer drug ellipticine in rats determined by ^{32}P-postlabeling. *Int. J. Cancer* **2003**; *107*, 885-890.
29. Stiborová, M.; Stiborová-Rupertová, M.; Bořek-Dohalská, L.; Wiessler, M.; Frei, E. Rat microsomes activating the anticancer drug ellipticine to species covalently binding to deoxyguanosine in DNA are a suitable model mimicking ellipticine bioactivation in humans. *Chem. Res. Toxicol.* **2003**, *16*, 38-47.
30. Stiborová, M.; Poljakova, J.; Martínková, E.; Ulrichová, J.; Šimánek, V.; Dvořák, Z.; Frei, E. Ellipticine oxidation and DNA adduct formation in human hepatocytes is catalyzed by human cytochromes P450 and enhanced by cytochrome b_5. *Toxicology* **2012,** *302*), 233-241.
31. Kotrbová, V.; Aimová, D.; Březinová, A.; Janouchová, K.; Poljaková, J.; Hodek, P.; Frei, E.; Stiborová, M. Cytochromes P450 reconstituted with NADPH:P450 reductase mimic the activating and detoxicating metabolism of the anticancer drug ellipticine in microsomes. *Neuro Endocrinol. Lett.* **2006**, *27*(Suppl. 2), 18-20.
32. Stiborova, M.; Poljakova, J.; Mrizova, I.; Borek-Dohalska, L; Eckschlager, T., Adam, V., Kizek, R., Frei, E. Expression levels of enzymes metabolizing an anticancer drug ellipticine determined by electromigration assays influence its cytotoxicity to cancer cells - A comparative study. *Int. J. Electrochem. Sci.* **2014,** *9*, 5675-5689 .
33. Poljaková, J.; Frei, E.; Gomez, J.E.; Aimová, D.; Eckschlager, T.; Hraběta, J.; Stiborová, M. DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. *Cancer Lett.* **2007**, *252*, 270-279.
34. Poljaková, J.; Eckschlager, T.; Hraběta, J.; Hřebačková, J.; Smutný, S.; Frei, E.; Martínek, V.; Kizek, R.; Stiborová, M. The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells. *Biochem. Pharmacol,* **2009**, *77*, 1466-1479.

35. Poljakova, J.; Hrebackova, J.; Dvořákova, M.; Moserova, M.; Eckschlager, T.; Hrabeta, J.; Göttlicherova, M.; Kope‚jtkova, B.; Frei, E.; Kizek, R.; Stiborova, M. Anticancer agent ellipticine combined with histone deacetylase inhibitors, valproic acid and trichostatin A, is an effective DNA damage strategy in human neuroblastoma. *Neuro Endocrinol. Lett.* **2011**, *32*(Suppl 1), 101-116.
36. Poljaková, J.; Eckschlager, T.; Kizek, R.; Frei, E.; Stiborová, M. Electrochemical determination of enzymes metabolizing ellipticine in thyroid cancer cells - a tool to explain the mechanism of ellipticine toxicity to these cells. *Int. J. Electrochem. Sci.* **2013**, *8*, 1573-1585.
37. Martinkova, E.; Dontenwill, M.; Frei, E.; Stiborová, M.. Cytotoxicity of and DNA adduct formation by ellipticine in human U87MG glioblastoma cancer cells. *Neuro Endocrinol. Lett.* **2009**, *30*(Suppl. 1), 60-66.
38. Poljaková, J.; Dračínský, M.; Frei, E.; Hudeček, J., Stiborová, M. The effect of pH on peroxidase-mediated oxidation of and DNA-adduct formation by ellipticine. *Collect. Czech. Chem. Commun.* **2006**, *71*, 1169-1185.
39. Stiborová, M.; Arlt, V.M.; Henderson, C.J.; Wolf, C.R.; Kotrbová, V.; Moserová, M.; Hudecek, J.; Phillips, D.H.; Frei, E. Role of hepatic cytochromes P450 in bioactivation of the anticancer drug ellipticine: studies with the hepatic NADPH:cytochrome P450 reductase null mouse. *Toxicol. Appl. Pharmacol.* **2008**, *226*, 318-327.
40. Stiborová, M.; Moserová, M.; Mrázová, B.; Kotrbová, V.; Frei, E. Role of cytochromes P450 and peroxidases in metabolism of the anticancer drug ellipticine: additional evidence of their contribution to ellipticine activation in rat liver, lung and kidney. *Neuro Endocrinol. Lett.* **2010**, *31*(Suppl. 2), 26-35.
41. Stiborová, M.; Eckschlager, T.; Poljaková, J.; Hraběta, J.; Adam, V.; Kizek, R.; Frei, E. The synergistic effects of DNA-targeted chemotherapeutics and histone deacetylase inhibitors as therapeutic strategies for cancer treatment. *Curr. Med. Chem.* **2012**, *19*, 4218-4238.
42. Vranová, I.; Moserová, M.; Hodek, P.; Kizek, R.; Frei, E.; Stiborová, M. The anticancer drug ellipticine induces cytochromes P450 1A1, 1A2 and 3A, cytochrome b_5 and NADPH:cytochrome P450 in rat liver, kidney and lung. *Int. J. Electrochem. Sci.* **2013**, *8*, 1586-1597.
43. Chadwick, M.; Silveira, D.M.; Platz, B.R.; Hayes, D. Comparative physiological disposition of ellipticine in several animal species after intravenous administration. *Drug Metab. Dispos.* **1978,** *6*, 528-541.
44. Branfam, A.R.; Bruni, R.J.; Reihold, V.N.; Silveira, D.M.; Chadwick, M.; Yesair, D.W. Characterization of metabolites of ellipticine in rat bile. *Drug Metab. Dispos.* **1978,** *6*, 542-548.
45. Hofle, G.; Glase, N.; Leibold, T.; Sefkow, M. Epothilone A-D and their thiazole-modified analogs as novel anticancer agents. *Pure Appl. Chem.* **1999**, *71*, 2019-2024.
46. Moserova, M.; Kotrbova, V.; Rupertova, M.; Naiman, K.; Hudecek, J.; Hodek, P.; Frei, E.; Stiborova, M. Isolation and partial characterization of the adduct formed by 13-

687 hydroxyellipticine with deoxyguanosine in DNA. *Neuro Endocrinol, Lett.* **2008**, *29*, 728-
688 732.
689 47. Martínek, V.; Sklenár, J.; Dracínsky, M.; Sulc, M.; Hofbauerová, K.; Bezouska, K.; Frei,
690 E.; Stiborová, M. Glycosylation protects proteins against free radicals generated from
691 toxic xenobiotics. *Toxicol. Sci,* **2010**, *117*, 59-74.
692 48. Donaghy, M.J.; Stanforch, S.P. Nucleophilic addition of fused benzimidazole *N*-oxide,
693 *Tetrahedron* **1999**, *55*, 1441-1448.
694 49. Stiborová, M.; Borek-Dohalská, L.; Aimová, D.; Kotrbová, V.; Kukacková, K.;
695 Janouchová, K.; Rupertová, M.; Ryslavá, H.; Hudecek, J.; Frei, E. Oxidation pattern of the
696 anticancer drug ellipticine by hepatic microsomes - similarity between human and rat
697 systems. *Gen. Physiol. Biophys.* **2006**, 25, 245-261.
698 50. Stiborova, M.; Cerna, V.; Moserova, M.; Arlt, V.M.; Frei, E. The effect of benzo[a]pyrene
699 on metabolic activation of anticancer drug ellipticine in mice. *Neuro Endocrinol. Lett.*
700 **2013,** *34* (Suppl 2), 43-54.
701 51. Henderson, C.J.; Otto, D.M.; Carrie, D.; Magnuson, M.A.; McLaren, A.W.; Rosewell, I.;
702 Wolf, C.R. Inactivation of the hepatic cytochrome P450 system by conditional deletion of
703 hepatic cytochrome P450 reductase. *J. Biol. Chem* . **2003,** *278*, 13480-13486.
704 52. Arlt, V.M.; Stiborova, M.; Henderson, C.J.; Osborne, M.R.; Bieler, C.A.; Frei, E.;
705 Martinek, V.; Sopko, B.; Wolf, C.R.; Schmeiser, H.H.; Phillips, D.H. Environmental
706 pollutant and potent mutagen 3-nitrobenzanthrone forms DNA adducts after reduction by
707 NAD(P)H:quinone oxidoreductase and conjugation by acetyltransferases and
708 sulfotransferases in human hepatic cytosols. *Cancer Res.* **2005,** *65*, 2644-2652.
709 53. Arlt, V.M.; Henderson, C.J.; Wolf, C.R.; Schmeiser, H.H.; Phillips, D.H.; Stiborova, M.
710 Bioactivation of 3-aminobenzanthrone, a human metabolite of the environmental pollutant
711 3-nitrobenzanthrone: evidence for DNA adduct formation mediated by cytochrome P450
712 enzymes and peroxidases. *Cancer Lett.* **2006,** *234*, 220-2231.
713 54. Arlt, V.M.; Stiborová, M.; Henderson, C.J.; Thiemann, M.; Frei, E.; Aimová, D.; Singh,
714 R.; Gamboa da Costa, G.; Schmitz, O.J.; Farmer, P.B.; Wolf, C.R.; Phillips, D.H.
715 Metabolic activation of benzo[a]pyrene *in vitro* by hepatic cytochrome P450 contrasts
716 with detoxification *in vivo*: experiments with hepatic cytochrome P450 reductase null
717 mice. *Carcinogenesis* **2008,** *29*, 656-665.
718 55. Arlt, V.M.; Poirier, M.C.; Sykes, S.E.; John, K.; Moserova, M.; Stiborova, M.; Wolf, C.R.;
719 Henderson, C.J.; Phillips, D.H. Exposure to benzo[a]pyrene of Hepatic Cytochrome P450
720 Reductase Null (HRN) and P450 Reductase Conditional Null (RCN) mice: Detection of
721 benzo[a]pyrene diol epoxide-DNA adducts by immunohistochemistry and ^{32}P-
722 postlabelling. *Toxicol. Lett.* **2012,** *213*, 160-166.
723 56. Pass, G.J.; Carrie, D.; Boylan, M.; Lorimore, S.; Wright, E.; Houston, B.; Henderson, C.J.;
724 Wolf, C.R. Role of hepatic cytochrome P450s in the pharmacokinetics and toxicity of
725 cyclophosphamide: studies with the hepatic cytochrome P450 reductase null mouse.
726 *Cancer Res.* **2005,** *65*, 4211-4217.

57. Xiao, Y.; Ge, M.; Xue, X.; Wang, H.; Wu, X.; Li, L.; Liu, L.; Qi, X.; Zhang, Y.; Li, Y.; Xie, T.; Gu, J.; Ren, J. Detoxication role of hepatic cytochrome P450s in the kidney toxicity induced by aristolochic acid. *Kidney Int.* **2008,** *73*, 1231-1239.
58. Levová, K.; Moserová, M.; Kotrbová, V.; Šulc, M.; Henderson, C.J.; Wolf, C.R.; Phillips, D.H.; Frei, E.; Schmeiser, H.H.; Mareš, J.; Arlt, V.M.; Stiborová, M. Role of cytochromes P450 1A1/2 in detoxication and activation of carcinogenic aristolochic acid I: studies with the hepatic NADPH:cytochrome P450 reductase null (HRN) mouse model. *Toxicol. Sci.* **2011,** *121*, 43-56.
59. Eling, T.E.; Thompson, D.C.; Foureman, G.L.; Curtis, J.F.; Hughes, M.F. Prostaglandin H synthase and xenobiotic oxidation. *Annu. Rev. Pharmacol. Toxicol.* **1990,** *30,* 1–45.
60. Eling, T.E.; Curtis, J.F. Xenobiotic metabolism by prostaglandin H synthase. Pharm. Ther. **1992***, 53,* 261–273.
61. Stiborova, M.; Frei, E.; Hodek, P.; Wiessler, M.; Schmeiser, H.H. Human hepatic and renal microsomes, cytochromes P450 1A1/2, NADPH:cytochrome P450 reductase and prostaglandin H synthase mediate the formation of aristolochic acid-DNA adducts found in patients with urothelial cancer. *Int. J. Cancer* **2005,** *113,* 189–197.
62. Downie, D.; McFadyen, M.C.; Rooney, P.H.; Cruickshank, M.E.; Parkin, D.E.; Miller, I.D.; Telfer, C.; Melvin, W.T.; Murray, G.I.. Profiling cytochrome P450 expression in ovarian cancer: identification of prognostic markers. *Clin. Cancer Res.* **2005,** *11,* 7369–7735.
63. Saarikoski, T.; Rivera, S.P.; Hankinson, O.; Husgafvel-Pursiainen, K. CYP2S1: a short review. *Toxicol. Appl. Pharmacol.* **2005,** *207,* 62–69.
64. Bui, P.H.; Hankinson, O. Functional characterization of human cytochrome P450 2S1 using a synthetic gene-expressed protein in *Escherichia coli*. *Mol. Pharmacol.* **2009,** ***76,*** 1031–1043.
65. Bui, P.H.; Hsu, E.L.; Hankinson, O. Fatty acid hydroperoxides support cytochrome P450 2S1-mediated bioactivation of benzo[a]pyrene-7,8-dihydrodiol. *Mol. Pharmacol.* **2009,** ***76,*** 1044–1052.
66. Mrizová, I.; Moserová, M.; Milichovsky, J.; Šulc, M.; Guengerich, F.P.; Stiborová, M. Heterologous expression of cytochrome P450 2S1 in *Escherichia coli*. *Interdisc. Toxicol.* **2014,** *7,* 66.
67. Stiborova, M.; Moserova, M.; Černá, V.; Indra, R.; Dračínský, M.; Šulc, M.; Henderson, C.J.; Wolf, C.R.; Schmeiser, H.H.; Phillips, D.H.; Frei, E.; Arlt, V.M. Cytochrome b_5 and epoxide hydrolase contribute to benzo[*a*]pyrene-DNA adduct formation catalyzed by cytochrome P450 1A1 under low NADPH:P450 oxidoreductase conditions. *Toxicology* **2014,** *318,* 1-12.
68. Rendic, S.; DiCarlo. F.J. Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab. Rev.* **1997,** 29, 413–480.
69. Ueng, Y.-F.; Kuwabara, T.; Chun, Y.-J.; Guengerich, F.P. Cooperativity in oxidation catalyzed by cytochrome P450 3A4. *Biochemistry* **1997,** *36,* 370–381.

70. Patterson, L.H.; McKeown, S.R.; Robson, T.; Gallagher, R.; Raleigh, S.M.; Orr, S. Antitumor prodrug development using cytochrome P450 (CYP) mediated activation. *Anti-Cancer Drug Design* **1999,** *14,* 473-486.
71. Murray, G.I.; Melvin, W.T.; Burke, M. Cytochrome P450 expression in tumors. *J. Pathol*. **1995,** *176,* 323-324.
72. El-Rayes, B.F.; Ali, S.; Heilbrun, L.K.; Lababidi, S.; Bouwman, S.; Vischer, D.; Philip, P.A. Cytochrome P450 and glutathione transferase expression in human breast cancer. *Clin. Cancer Res.* **2003,** *9,* 1705-1709.
73. Yamazaki, H.; Gillam, E.M.; Dong, M.S.; Johnson, W.W.; Guengerich, F.P.; Shimada, T. Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with cytochrome P450 3A4 and other forms: effects of cytochrome P450-P450 and cytochrome P450-b_5 interactions. *Arch. Biochem. Biophys,* **1997**, *342*, 329-337.
74. Yamazaki, H.; Shimada, T.; Martin, M.V.; Guengerich, F.P. Stimulation of cytochrome P450 reactions by apo-cytochrome b_5: evidence against transfer of heme from cytochrome P450 3A4 to apo-cytochrome b_5 or heme oxygenase. *J. Biol. Chem.* **2001**, *276*, 30885-30891.
75. Schenkman, J.B.; Jansson, I. The many roles of cytochrome b_5. *Pharmacol. Ther.* **2003**, *97*, 139-152.
76. Zhang, H.; Myshkin, E.; Waskell, L. Role of cytochrome b_5 in catalysis by cytochrome P450 2B4. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 499-506.
77. Zhang, H.; Im, S.C.; Waskell, L. Cytochrome b_5 increases the rate of product formation by cytochrome P450 2B4 and competes with cytochrome P450 reductase for a binding site on cytochrome P450 2B4. *J. Biol. Chem.* **2007**, *282*, 29766-29776.
78. Kotrbová, V.; Aimová, D.; Ingr, M.; Borek-Dohalská, L.; Martínek, V.; Stiborová, M. Preparation of a biologically active apo-cytochrome b_5 *via* heterologous expression in *Escherichia coli*. *Protein Expr. Purif.* **2009**, *66*, 203-209.
79. Rendic, S.; Guengerich, F.P. Contributions of human enzymes in carcinogen metabolism. *Chem. Res. Toxicol.* **2012**, *25*, 1316-1383.
80. Aimová, D.; Svobodová, L.; Kotrbová, V.; Mrázová, B.; Hodek, P.; Hudeček, J.; Václavíková, R.; Frei, E.; Stiborová, M. The anticancer drug ellipticine is a potent inducer of rat cytochromes P450 1A1 and 1A2, thereby modulating its own metabolism. *Drug Metab. Dispos.* **2007**, *35*, 1926-1934.
81. Godschalk, R.W.L.; Moonen, E.J.C.; Schilderman, P.A.E.L.; Broekmans, W.M.R.; Kleinjans, J.C.S.; van Schooten, F.J. Exposure-route-dependent DNA adduct formation by polycyclic aromatic hydrocarbons. *Carcinogenesis* **2000,** *21,* 87-92.
82. Poirier, M.C. Chemical-induced DNA damage and human cancer risk. *Nature Reviews* **2004,** *4,* 630-637.
83. Randerath, K.; Haglund, R.E.; Phillips, D.H.; Reddy, M.V. ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis* **1984,** *5,* 1613-1622.

84. Ross, J.A.; Nelson, G.B.; Wilson, K.H.; Rabinowitz, J.R.; Galati, A.; Stoner, G.D.; Nesnow, S.; Mass, M.J. Adenomas induced by polycyclic aromatic hydrocarbons in strain A/J mouse lung correlate with time-integrated DNA adduct levels. *Cancer Res.* **1995,** *55,* 1039-1044.
85. Smith, B.A.; Fullerton, N.F.; Heflich, R.H.; Beland, F.A. DNA adduct formation and T-lymphocyte mutation induction in F344 rats implanted with tumorigenic doses of 1,6-dinitropyrene. *Cancer Res.* **1995,** *55,* 2316-2324.
86. Finn, R.D.; McLaughlin, L.A.; Ronseaux, S.; Rosewell, I.; Houston, J. B.; Henderson, C.J.; Wolf, C.R. Defining the *in vivo* role for cytochrome b_5 in cytochrome P450 function through the conditional hepatic deletion of microsomal cytochrome b_5. *J. Biol. Chem.* **2008,** *283*, 31385-31393.
87. Henderson, C.J.; McLaughlin, L.A.; Wolf, C.R. Evidence that cytochrome b_5 and cytochrome b_5 reductase can act as sole electron donors to the hepatic cytochrome P450 system. *Mol. Pharmacol.* **2013,** *83*, 1209-1217.
88. Ma, J.; Waxman, D.J. Collaboration between hepatic and intratumoral prodrug activation in a P450 prodrug-activation gene therapy model for cancer treatment. *Mol. Cancer Ther.* **2007,** *6,* 2879-2890.
89. Lu, H.; Chen, C.S.; Waxman, D.J. Potentiation of methoxymorpholinyl doxorubicin antitumor activity by P450 3A4 gene transfer. *Cancer Gene Ther.* **2009,** *16,* 393-404.